Daun02 inactivation of behaviorally-activated Fos-expressing neuronal ensembles

Article (Accepted Version)

CURRENT PROTOCOLS
The Fine Art of Experimentation

Daun02 inactivation of behaviorally-activated Fos-expressing neuronal ensembles

Author(s) Eisuke Koya[1a], Gabriella Margetts-Smith[1b], and Bruce T. Hope[2*]

Contact information

1a. School of Psychology, University of Sussex, Falmer, BN1 9QG, United Kingdom; Tel: +44-1273-877-776; email: e.koya@sussex.ac.uk

1b. School of Psychology, University of Sussex, Falmer, BN1 9QG, United Kingdom; Tel: +44-1273-87-2952; email: gm238@sussex.ac.uk

2. NIDA IRP, NIDA/NIH/DHHS, Baltimore, MD 21224, USA; Tel: +1-443-740-2585; email: bhope@intra.nida.nih.gov

ABSTRACT Learned associations about salient experiences (e.g. drug exposure, stress) and their associated environmental stimuli are mediated by a minority of sparsely distributed, behaviorally activated neurons coined 'neuronal ensembles'. For many years, it was not known whether these neuronal ensembles played causal roles in mediating learned behaviors. However, in the last several years the 'Daun02 inactivation technique' in *Fos-lacZ* transgenic rats has proved very useful in establishing causal links between neuronal ensembles that express the activity-regulated protein 'Fos' and learned behaviors. Fos-expressing neurons in these rats also express the bacterial protein β-galactosidase (β-gal) in strongly activated neurons. When the prodrug Daun02 is injected into the brains of these rats 90 min after a behavior (e.g. drug-seeking) or cue exposure, then the Daun02 is converted into daunorubicin by β-gal, which selectively inactivates the Fos and β-gal-expressing neurons that were activated 90 min before the Daun02 injection. This unit presents protocols for breeding the *Fos-lacZ* rats and conducting appropriate Daun02 inactivation experiments.

Significance Statement

Learned behaviors are mediated by specific patterns of sparsely distributed neurons, called 'neuronal ensembles', that are selectively activated during behavior and cue exposures. For many years, neuroscientists observed that neural activity of specific neurons correlated with specific behaviors or cue exposures. However, they could not determine whether these selectively activated neurons play 'causal roles' in the learned behaviors because tools to *selectively* silence only the behaviorally activated neurons without affecting the surrounding neurons were unavailable. The Daun02 inactivation technique described in this protocol selectively inactivates neurons that were activated strongly enough during recent behavior to express the proteins β-galactosidase (β-gal) in *Fos-lacZ* transgenic rats. Researchers use this tool to establish whether specific neuronal ensembles directly mediate learned behaviors.

Keywords: neuronal ensemble inactivation, Daun02 inactivation, *Fos-lacZ* rodents

INTRODUCTION For many decades, neuroscientists have been deciphering the neurobiological basis of learned behaviors by mapping the activity patterns of neurons. Early studies from the 1970's using *in vivo* electrophysiology tools have observed that only a minority of neurons were activated during learned behaviors (John and Schwartz, 1978; Umemoto and Olds, 1975). Around the 1980-90's, researchers complemented these electrophysiology findings using immediate early gene (IEG) markers of strong neuronal activity (Guzowski, 2002; Herdegen and Leah, 1998; Morgan and Curran, 1988; Morgan and Curran, 1991). *Fos* mRNA and its protein product Fos (Morgan and Curran, 1988; Morgan and Curran, 1991), a commonly used IEG marker, have been used to perform large-scale activity mapping of many brain areas in behavior. Today, it is known that various stimuli induce Fos in motivationally relevant brain areas, including the prefrontal cortex and nucleus accumbens (Cruz et al., 2014b). These stimuli range from stressful stimuli, drugs of abuse (e.g. cocaine), ingestion of palatable foods, and cues associated with drug and natural rewards (Cruz et al., 2013). Although interesting, these findings were purely correlative and did not demonstrate whether these activated neurons played a direct causal role in the learned behaviors. Until recently, tools did not exist to selectively silence the small number of activated neurons without disrupting activity of the surrounding majority of neurons.

To overcome this challenge, we developed a tool, coined the 'Daun02 inactivation procedure', to selectively silence Fos-expressing neurons (Fig. 1) and demonstrate a causal for Fos-expressing ensembles in a behavior (Koya et al., 2009). In this inactivation procedure, *Fos-lacZ* transgenic rats undergo some form of learning, and then are exposed to external stimuli (e.g. reward-related cues) that reactivate a neuronal ensemble and induce expression of the activity-dependent transgene *lacZ* and its protein product β-gal in the activated Fos-expressing neurons (Bossert et al., 2011; Koya et al., 2009). When β-gal levels are maximal, the inactive prodrug Daun02 (Farquhar et al., 2002) is injected directly into the brain area of interest. Daun02 is converted by β-gal into daunorubicin to reduce cellular excitability (Engeln et al., 2014) and induce apoptosis and cell death (Pfarr et al., 2015). Thus, activated β-gal expressing neurons are selectively and persistently silenced, while the surrounding neurons are unaffected. The behavioral consequences of inactivating the Fos/β-gal-expressing neuronal ensembles can then be examined.

This unit describes the Daun02 inactivation procedure that allows inactivation of behaviorally-activated β-gal (and Fos)-expressing neurons in *Fos-lacZ* rats. Although *Fos-lacZ* mice that express β-gal in activated neurons have also been generated (Smeyne et al., 1992), our focus here will be on the *Fos-lacZ* rat, since to date there are no published studies that use Daun02 inactivation in these mice. There are multiple Basic Protocols in this unit with several key steps described in each Protocol, and they are written in the order of the events you will encounter from start to finish. Figure 2 provides a general overview of the major steps.

Note: All protocols using live animals must be reviewed first by institutional ethical committees (and/or any other government regulatory agencies in your country) and must follow officially approved procedures for care and use of laboratory animals.

BASIC PROTOCOL 1

Breeding and genotyping of *Fos-lacZ* transgenic rats

Fos-lacZ rats are bred on a Sprague-Dawley background. Use only hemizygous *Fos-lacZ* rats. We found that homozygous *Fos-lacZ* rats express higher basal levels of β-gal that will interfere with selective Daun02 inactivation of only the behaviorally-activated neurons (unpublished observations). An animal hemizygous for the *Fos-lacZ* gene (male or female) should be mated with an outbred wild-type animal (male or female). Approximately 50% of the offspring will be transgene-positive. As a general rule of thumb, 11-12 breeding pairs will produce approximately 28-32 positive male and female offspring per month. Ear punches or tail snips can be used for genotyping. The PCR protocol, including specific primer sets, for detecting the *Fos-lacZ* gene is provided below, but the protocol can be modified depending on the primer set.

Materials

Ear punch collector (for ear punches) (Cat no. 24212-02, Fine Science Tools)

Local (e.g. lidocaine cream) or general anesthetic (e.g. isoflurane) (for tail tip removal)

Sterile blade or scissors

1.5mL microfuge tubes

Vortex device

Sodium hydroxide solution 10.0M (Cat no. 72068-100ML, Sigma)

Trizma® hydrochloride solution 1M (Tris-HCl) pH 8 (Cat no., T2694-1L, Sigma)

OneTaq DNA polymerase Mastermix (Cat no. M0482S, New England Biolabs)

Oligos (IDT DNA Technologies, standard synthesis, and desalting preparation)

PCR primers forward sequence: GTTGCAGTGCACGGCAGATACACTTGCTGA ; reverse sequence GCCACTGGTGTGGGCCATAATTCAATTCGC

100bp DNA ladder (Cat no. N3231S, New England Biolabs)

Ultra pure 10x TAE buffer (Cat no. 15558-026, ThermoFisher Scientific)

Agarose (for DNA gel electrophoresis)

Ethidium bromide

Sub-Cell® Model 96 Cell and PowerPac™ Universal Power Supply (Bio-rad) or equivalent

Protocol steps

1. Obtain tissue samples for genotyping. For ear punches, gently restrain the rat and remove a punch using an ear punch collector. A tail sample from the rat may also be removed for genotyping purposes, but this procedure is more invasive and it will require the appropriate local or general anesthetic if the rat is more than 3.5 weeks old. Clean the tail with alcohol first and use a sterile blade or scissors to remove the distal 2-4 mm portion of the tail. Remember to disinfect the blade or scissors (e.g. using hot beads) between rats. Mild anesthesia (e.g. isoflurane) can be used for either ear punches or tail samples. Place the

biopsy sample in a 1.5ml microfuge tube. After the rat is returned to the cage, monitor the rat and use appropriate cauterizing agents (e.g. silver nitrate) for hemostasis if necessary. More information can be found here:
http://oacu.od.nih.gov/ARAC/documents/Rodent_Genotyping.pdf

2. Add 300 µL of 50mM NaOH.

3. Incubate tubes at 95^{o}C for 60 minutes.

4. Vortex tubes on medium power setting for 5 seconds.

5. Quick spin the tubes to bring down the condensation.

6. Neutralize each sample by adding 30 µL of 1M Tris-HCl (pH 8).

7. Vortex tubes on medium power setting for 5 seconds.

8. Quick spin the tubes to bring down the condensation. The undigested remnant of the sample or debris may remain visible at the bottom of the tube. Some debris is to be expected, and make sure to only take the supernatant (what we refer to as 'genomic DNA lysate') when setting up the PCR reaction.

9. Add 1µL of genomic DNA lysate to the PCR 'master mix' (see Reagents and Solutions section).

10. For the PCR reaction use the following reaction conditions:

0. 94 ^{o}C – HOLD (hot start)

1. 94 ^{o}C – 2 min

2. 94 ^{o}C – 30 sec

3. 68 ^{o}C – 1 min

4. Go to step 2, repeat 40x

5. 4 ^{o}C – HOLD (end of program)

11. Analyze the PCR reaction products by electrophoresis in a 2% agarose gel in 1x TAE buffer. The addition of 0.5 µg of ethidium bromide per 1 mL of agarose solution will indicate the position of the PCR products.

BASIC PROTOCOL 2

Preparation of Daun02

Daun02 (MW 884.79) is an anthracyline-based 'prodrug' that is initially biologically inactive. Daun02 becomes biologically active when it is converted into daunorubicin (also known as

daunomycin) by the bacterial protein β-gal. In the first study, Daun02 was solubilized in 50% DMSO and 50% artificial cerebrospinal fluid (aCSF), which worked well for nucleus accumbens injections (Koya et al., 2009). For reasons unknown, this vehicle produces more neuronal damage in cortical areas, and thus we and others routinely use 4 μg/μL Daun02 in 5% DMSO and 6% Tween-80 in PBS, which can be prepared using the protocol below.

Note: The Daun02 compound is available from several manufacturers and should be stored at -20 ^{o}C in a sealed container with desiccant. As with all anthracyline-related compounds, it should be handled in a well-ventilated area using appropriate safety measures (e.g. gloves). Also, when dissolving Daun02 make sure to use containers using DMSO compatible material (e.g. LDPE, HDPE, polypropylene, PPCO, polymethylpentene) that tend to have an opaque colour, as non-compatible materials (e.g. polysulfone, PVC, polycarbonate) with a clear appearance, will be dissolved by DMSO.

Materials

Daun02 (Cat no. SRP04000g; Sequoia Research Products)

Dimethyl sulfoxide (DMSO) (Cat no. D8418-50mL, Sigma)

Tween-80 (Cat no. P4780-100mL, Sigma)

10x Phosphate buffered saline (PBS) (Cat no. P5493-1L, Sigma)

Artificial cerebral spinal fluid (aCSF) (Cat No. 3525, Tocris)

1. Dissolve Daun02 in 100% DMSO in a microfuge tube at a concentration of 80 μg/μL by gently pipetting up and down, followed by vortexing. This stock solution may be stored at -20^{o}C for up to 1 year (or at -80^{o}C for several years).

2. On the day of use, fully thaw the frozen aliquot to room temperature or if freshly prepared, directly dissolve 2.5 μL of 80 μg/μL Daun02 in DMSO (or 2.5 μL DMSO to make vehicle) for every 15 μL of 20% Tween-80. Make 9 μL aliquots and store at -20^{o}C for up to 1 year or -80^{o}C for several years.

3. On the day of use, fully thaw frozen Daun02 (or vehicle) aliquot to room temperature. Then add 16.5 μL of sterile PBS or aCSF and mix well to make a 4 μg/μL Daun02 solution in 5% DMSO, 6% Tween-80 vehicle.

   NOTE: Daun02 will precipitate irreversibly if PBS or aCSF is added before the Daun02 stock solution has warmed to room temperature. It is important to make the vehicle control for injections using the same solution.

4. Quick spin both the Daun02 and vehicle to bring solution to the bottom of the microfuge tube. Keep this solution at room temperature until use, and discard the remaining solution.

BASIC PROTOCOL 3

Surgical procedures for guide cannula implantation for rat

In order to exert its neuronal inactivation functions in a region-specific manner, Daun02 must be directly injected into the target brain areas, since anthracycline-related compounds do not effectively cross the blood-brain barrier (Rousselle et al., 2000). Thus, *Fos-lacZ* rats must be intracranially implanted with guide cannula prior to Daun02 infusions. This protocol describes this cannula implantation. For a useful video guide, please see (Fornari et al., 2012).

Materials

Any anesthesia (e.g. isoflurane/air mixture or ketamine and xylazine) that will last for the duration of surgery (approximately 1 hour).

Atropine sulfate

Betadine disinfectant

70% ethanol

Dental acrylic (Plastics one)

Stereotaxic atlas for rat

Stereotaxic frame equipped with carrier for guide cannula (David Kopf Instruments, Stoelting)

26-G internal cannula (Plastics one)

Shaver

Scalpel

Serrefine forceps or hemostats

Cotton Swabs

Dental cotton rolls

Metal Spatula

Dental drill

Bone screws: 4.8mm shaft length, 2.5mm head diameter, 1.57mm shaft diameter (PART# 0-80 x 3/16; Plastics One)

Jeweler's forceps and screwdriver

Protocol steps

1. Using a stereotaxic brain atlas such as (Paxinos, 1986) to determine the stereotaxic coordinates for the target brain areas. Make sure to order or make the correct lengths of guide cannulas and injector needles, so that the needles protrude 0.5-1 mm beyond the tip of the guide cannulas. This protrusion ensures that the Daun02 is properly delivered to the target area. A commonly used landmark on the skull is the

'bregma' where the major fissures of the skull form a plus-like symbol. This examination should be done after the anesthetized animal has been inserted into the stereotaxic frame. Pilot studies are highly recommended for accurate guide cannula placement since the actual injection sites may differ from the 'intended' sites.

2. Make sure to sterilize all surgical instruments via autoclaving or chemical sterilization. Continue to use aseptic conditions during surgeries.

3. Once the rat is properly restrained, inject it with an anesthetic that will last for the duration of surgery (e.g. intraperitoneal injections of ketamine and xylazine or inhaled isoflurane/air mixture). If necessary, administer atropine to relieve respiratory distress. The efficacy of the anesthesia should be monitored by observing the breathing (which should mostly be abdominal) and test for pain reflexes by pinching on of the feet with forceps or using your fingers. If a response is observed after 10 min, a supplemental dose of anesthetic should be administered.

4. Shave the fur off the rat's head until the surface is smooth. After checking again that the rat is deeply anesthetized and there is no pain reflex (e.g. following pinching the rear foot), mount the rat in the ear bars of the stereotaxic apparatus. Read the coordinates of each ear bar and center the animal between the bars. Open the rat's mouth and insert the upper incisors over the incisor bar. Adjust the nose clamp to firmly hold the rat's head.

5. Disinfect the head using Betadine, followed by 70% ethanol, and then Betadine again. Make an incision around the midline of the head using a scalpel using one smooth stroke. Then retract the skin using hemostats or Serrafine forceps.

6. Expose the skull, by first making an incision using a sterile scalpel and scrape off the dura matter using a metal spatula. Clean off any excess blood using cotton swabs or dental cotton rolls. Lower the guide cannula so that it is centered on the bregma, which is the intersection of the coronal and sagittal sutures. Read the anterior-posterior (AP), lateral (L), and dorsal-ventral (DV) coordinates from the scale, and add or subtract from the bregma coordinates to calculate the relative coordinates for the intended injection site.

7. Move the guide cannula to the intended anterior-posterior and lateral coordinates on the skull surface to identify the drilling sites. You may use a fine-tipped felt pen to mark the drilling point. Then raise the stereotaxic arms so that the skull may be drilled.

Implantation of the guide cannula

8. Drill two holes bilaterally into the skull where the guide cannulae are to be inserted. Use a hand drill with a drill bit slightly larger than the diameter of the guide cannula. Drill four more small holes for bone screws with two holes in each of the parietal and frontal bones of the left and right hemispheres. Make sure that these holes are not too close to the guide cannula holes and do not drill through the skull fissures. Drill slowly and carefully until the dura is broken, but do not damage the brain tissue by drilling too deep.

9. Using jeweler's forceps and a screwdriver, insert the 3/16-inch bone screws into the four smaller holes.

10. Attach the guide cannula into the stereotaxic holder and straighten. Carefully lower the guide cannula into the target area until the cannula base rests on the skull.

11. Dry the skull again if necessary and remove the skin retractors. It is important that the skull is dry in order for the dental cement to adhere to the skull. Mix dental acrylic and liquid component until the mixture is wet. While it is wet, apply this mixture to the skull around the base of the guide cannula and screws.

12. Once the acrylic is dry, remove the stereotaxic holder. If there are any jagged edges, make sure to smooth them out by applying additional dental acrylic mixture. These jagged edges will irritate the rat.

13. Remove the rat from the stereotaxic holder, and implant a dummy cannula into the guide cannula. Allow the rat to rest in its home cage and use a heating blanket and thermometer to maintain proper body temperature.

14. The rats should be allowed to recover for 7 days (or longer if necessary) before commencing any behavioral experiments. Make sure to unblock the guide cannula for debris every 2 days by repeatedly inserting the dummy cannula up and down.

BASIC PROTOCOL 4

Intracranial infusions of Daun02 and subsequent behavioral testing

Behavioral training may commence approximately a week after surgery. Many Daun02 studies to date have silenced neurons that were activated during different motivated behaviors on 'induction day', and then tested 3 days later to observe whether the behavior of interest was disrupted (Bossert et al., 2011; Cruz et al., 2014a; Fanous et al., 2012; Koya et al., 2009). On induction day, we reactivate a specific neuronal ensemble by exposing the rat to cues associated with the learned behavior, with or without performance of the actual behavior, to induce β-gal selectively within the relevant ensemble 90 min prior Daun02 injections. This protocol describes how and when to intracranially infuse Daun02 to silence the neuronal ensembles of interest on induction day. It is highly advisable to perform pilot experiments to see if the target brain area expresses β-gal and characterize the degree of neuronal activation (i.e. counting the number of Fos or β-gal expressing neurons) during the behavior of interest, to gain better insight about the nature of the neuronal ensemble that is recruited.

Materials

Fos-lacZ rat with implanted guide cannula

Daun02 (prepared from previous protocol, 4µg/µL at room temperature)

Vehicle (prepared from previous protocol)

Flexible tubing

Hamilton syringes (10µL, with 30 G needle)

Infusion pump (Item 702001; Harvard Apparatus)

1. Two to three days before induction day, use dummy injector needles that are shorter than the guide cannula to practice a sham infusions session. The purpose of this step is to habituate the animals to the infusion procedure, and to unclog the cannula in preparation for injections.

2. On induction day, prepare the Daun02 solution as described in protocol 2 step 3 for a final concentration of 4 µg/µL. Attach plastic tubing to 2 Hamilton syringes, then attach 2 injector needles. Make sure that the tubing and needles are cleaned first with 70% ethanol, then water. Push out excess water, and then aspirate 10 µL of Daun02 or vehicle using the Hamilton syringes. Then use the pump to push the Daun02 or vehicle solution until it is slightly dripping from the injector needles. Wipe off excess fluid with sterile gauze.

3. Initiate the behavior or cue exposure to induce β-gal in activated neurons. β-gal expression is at peak level 90 min after initiation of the behavioral test. Perform injections in a quiet room because rats can be extra sensitive during this time. Gently restrain the rat and insert both injector needles into the bilateral cannulas. Place the rat in a comfortable cage with bedding while infusing 0.5 µL of Daun02 or vehicle at a rate of 1 µL/min. Once the infusions are over, the injector needles should remain in the animal for an additional 1 min.

   To date, many behavioral studies have used relatively short behavioral tests (e.g. 15-30 min) since they utilized procedures in which extinction learning took place (e.g. lever-pressing for a drug-associated cue).(Bossert et al., 2011; Cruz et al., 2014a; Fanous et al., 2012) *The duration was kept short to prevent the behavior of interest from being fully extinguished so that the behavior could still be observed on test day. As a general guideline, a minimum and maximum test duration of 15 and 30 min will allow the behavior of interest to be adequately measured, while reducing the likelihood of it being extinguished. However, pilot experiments are recommended to determine the ideal test duration that produces a sufficient level of responding on the Daun02 infusion day and the final test day for your behavior of interest. Finally, it is important to include proper control groups in demonstrate that Daun02 effects are cue- or context-specific. Usually these control groups are exposed to stimuli (e.g. novel, neutral context) that would likely activate a neuronal ensemble distinct from the neuronal ensemble of interest.*

4. The behavioral test is performed 3 days later to assess the effects of Daun02 versus vehicle injections. Recent evidence suggests that this time point allows Daun02 sufficient time to inactivate β-gal-expressing neurons. Although we and others normally test the effects of Daun02 three days following its infusion, it may be possible that Daun02 is efficacious at time points earlier or later than 3 days. Users may want to perform pilot experiment to examine whether this is the case.

5. 90-120 min after initiating the behavioral test, users may choose to transcardially perfuse the rats using 4% paraformaldehyde for X-gal staining or immunohistochemistry of brain sections to assess Daun02-mediated decreases of activated neurons using X-Gal staining of β-gal (see next protocol) or immunohistochemistry for Fos protein. Alternatively, since Daun02 has been shown to permanently silence neurons via apoptosis, one may also continue to test the rats on other behavioral tests to examine the effects of this inactivation.

BASIC PROTOCOL 5

Transcardial perfusions for X-gal staining (or immunohistochemistry for β-gal or Fos) following the final behavioral test.

In previous Daun02 studies, the numbers of β-gal-expressing neurons were quantified following the final behavioral test in order to determine the degree of inactivation of cue- or context-activated neurons (Bossert et al., 2011; Cruz et al., 2014a; Fanous et al., 2012; Koya et al., 2009). The protocol below describes how to transcardially perfuse a *Fos-lacZ* rat following a behavioral test to prepare the brain tissue for X-gal staining or β-gal (or Fos) immunohistochemistry**.** Although both types of staining can be used to detect changes in the number of strongly activated neurons, the advantage of X-gal staining is that it has a very high signal to noise ratio.

Materials

Peristaltic pump attached to an IV line with 16 gauge blunt end needles at end (for rat)

Paraformaldehyde (PFA, for making 4% PFA) (Cat no. P6148-1KG, Sigma)

Rat guillotine (Cat no. DCAP, Kent Scientific Corportation)

Sucrose (for making 30% sucrose solution in PBS)

10x PBS pH 7.4 for making (1x PBS) (Cat no. P5493-1L, Sigma)

Sodium hydroxide solution 10.0 M (Cat no. 72068-100ML, Sigma)

Sodium phosphate monobasic monohydrate (Cat no. S9638-25G, Sigma)

Sodium Chloride (Cat no. S7653-1kg, Sigma)

Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Cat no. E3889-25G)

Magnesium chloride solution, 1.0M (Cat no. M1028-100mL, Sigma)

Triton X-100 (Cat no. 93443-100mL, Sigma)

Potassium ferricyanide (III) $K_3Fe(CN)_6$ (Cat no. 244023-100G, Sigma)

Potassium hexa-cyanoferrate (II) trihydrate $K_4Fe(CN)_6 \bullet 3H_2O$ (Cat no. P3289-100g, Sigma)

5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Cat no., B71800-1.0, RPI corporation)

N,N-Dimethylformamide (DMF) (Cat no., D4551-250mL, Sigma)

Chrome-Alum coated glass slides (or any other electrostatically charged slides, e.g. SuperFrost Plus slides (Catalog no. 22-037-246, Fisher Scientific))

Corning® Costar® cell culture plates 6 well, (Cat no. CLS3516-50EA, Sigma)

Corning® Netwells® inserts, membrane diam. 24 mm (Cat no. CLS3480-48EA, Sigma)

Staining racks and handles (Cat. No. 70312-24, 70312-25, Electron microscopy services)

6 Staining dishes (Cat. No 70312-20, Electron microscopy services)

CitriSolv™ Solvent and Clearing Agent *(Cat no. 89426-268, VWR)*
(In the UK use Histoclear III (Cat no. HS-204-1 gal, AGTC Bioproducts)

Fisher Chemical Permount™ Mounting Medium (Cat no. SP15-500, Fisher)

(In the UK use Histomount (Cat no. HS-103-100mL, AGTC Bioproducts)

Incubator or waterbath

Any anesthetic that will produce deep anesthesia (e.g. isoflurane saturated in air).

Protocol steps

Transcardial perfusions

1. Prepare 1x PBS, 4% paraformaldehyde (PFA), and 30% sucrose (in PBS) solutions. These should be set up together with the perfusion equipment before the behavioral testing begins. Prepare approximately 100 of PBS and 500 mL of 4% PFA per rat.

2. Deeply anesthetize the rat 90-120 min following initiation of the behavioral test. This is easily done by placing the rat in a glass dessicator for 90 sec with isoflurane saturated in air. After confirming that the animal is deeply anesthetized by checking for the pain reflex (e.g. following pressure application on the rear foot), use a large pair of scissors to cut horizontally through the skin and muscle of the thorax just below the xiphoid process, which can be identified by pressing your finger along the mid-axillary line.

   As with all perfusions, try to reduce the time between opening up the rat and perfusing PBS. Blood will begin to clot if blood stops flowing and compromise subsequent labeling (particularly immunolabelling) of activated neurons. It should be relatively easy to keep this time down to 2-3 minute or less with practice.

3. Using a hemostat, grasp the now exposed xiphoid process and raise the anterior body wall. Cut through the abdominal muscles along the inferior margin of the ribs.

4. Cut through the diaphragm along its attachment to the ventral and lateral margins of the rib cage. Be careful not to damage the heart that lies above the diaphragm.

5. Cut through the lateral part of the rib cage on both sides up to the collar bone. Use the hemostat attached to the xiphoid process to retract the sternum towards the rat's head. This should provide a clear view of the heart and vessels. The heart should be beating at this stage. Remove any connective tissue around the heart.

6. Using small scissors, make a transverse cut through the posterior left aspect of the heart to open the rat's left ventricle (at the vertex of the heart). While gently clamping the heart using forceps, insert the perfusion needle through the opening in the left ventricle into the ascending aorta. Clamp the cannula in place using a hemostat to secure the position of the needle. The hemostat should be clamped around the heart tissue immediately below the descending aorta.

7. After confirming the placement of the needle tip, make a small incision in the right atrium to make an outlet. Then turn on the perfusion pump and perfuse PBS into the animal at a flow rate of 30 mL/min. Once PBS has replaced the blood in the animal's vascular system, and the fluid escaping the atrium is clear, then transfer the pump input line from the PBS to 4% PFA solution.

   If fluid is coming out of the nostrils, then reduce the rate of perfusion. This effect is due to back flow of perfusion fluid into the lungs, and often due to placement of the tip of the perfusion needle too low down in the ventricle rather than in the ascending aorta.

8. Once the transfer of PBS to PFA tubing is complete, begin pumping the 4% PFA solution. As the fixative solution enters the body, the body should rapidly begin to stiffen and the rat will appear to be shaking due to muscle contractions. Perfuse the desired volume, turn off the pump and remove the cannula from the heart.

9. Clear the line of 4% PFA, and refill it with PBS again to commence the next perfusion. Make sure all the lines are clear of air bubbles.

10. Decapitate the rat's head using a guillotine. Remove the skin on top of the head and expose the skull. Use Rongeurs to remove small bits of the skull around the foramen magnum, and use scissors to make a small incision around the midline of the skull around the occipital bone. Using the Rongeurs, very carefully remove the occipital, parietal, and frontal bones. Once the brain is fully exposed, remove the brain using a spatula by gently going underneath the brain and elevating the brainstem until the optic nerves pull free. Post-fix the brain by immersing it in 4% PFA for 1-4 hours. Transfer the brain to a small container (e.g. 50 ml conical tube) containing 30% sucrose solution diluted in PBS. The brains should remain in this sucrose solution at 4^{o}C until they sink to the bottom (which may take 2-3 days). Brains may remain in sucrose for up to five days. Once sunk, the brains can be frozen in hammer-crushed dry ice for at least 45 min, then wrapped in aluminum foil (to prevent dehydration), and stored at -80^{o}C overnight (or until further use).

    It is important not to overfix the brain (e.g. overnight in 4% PFA) as this might decrease the action of β-gal enzyme and hence the efficacy of the X-gal staining procedure (that is dependent on β-gal enzymatic activity) (Ma et al., 2002).

11. Mount the brain onto a cryostat and cut 30-40 µm brain sections in a cryostat and collect the sections in PBS (with 0.02% w/v Sodium azide) at RT. While slicing, pay close attention to the location of the injection sites and make sure to collect these sections. Sections may be stored at 4°C for 1-2 weeks or may be used immediately for X-gal staining. Alternatively, sections may be stored in cryopreservant at -80°C until further use.

X-gal histochemistry

12. Wash sections 3 x 10 min in PBS using 6-well plates and net wells.

13. Incubate sections in X-gal solution in a 6-well plate in an incubator (or 1.5 mL tubes in water bath away from light) for 2-3 hours (or over night if β-gal is to be detected in naïve animals) at 37°C while gentle shaking.

14. Wash sections 3 x 10 min in PBS

15. Mount onto chrom-alum coated slides and dry over night.

16. Place slides in increasing concentrations of ethanol (2 min each at 70%, 80%, 95%, 100%, 100% ethanol, followed by 10 min of CitriSolv Solvent or Histoclear clearing agent). Remove excess solvent and coverslip using mounting medium (Permount or Histomount). Allow the mounting medium to dry over night before imaging.

17. Observe β-gal labeling using a bright field microscope. Due to the extremely low background the tissue may appear almost translucent and it might be difficult to determine some major anatomical landmarks in X-gal stained brained sections to properly identify the region of interest. This problem may be overcome by 1) Illuminating your sample using a fluorescent light source; 2) Creating more contrast in the section by adjusting the amount of light coming in using the iris. Both methods help visualize the brain section by providing more contrast and thus making major anatomical landmarks more visible. Properly labeled nuclei should have a dark blue appearance (see Fig. 3) In order to determine Daun02-mediated decreases in activated neurons, count β-gal-positive cells that are 200-300 µm around the injection site from 2-3 sections from Daun02 and vehicle-injected animals.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes.

PCR mix

Recipe for 10 µL: Add 5.5 µL of 2x One Taq Mastermix, 1.1 µL of 5 µM forward and 5 µM reverse primers and 3.4 µL of water.

Transgene-specific oligos for OTTC126 (lacZ gene):

Forward primer	LacZ F2538	GTTGCAGTGCACGGCAGATACACTTGCTGA
Reverse primer	LacZ R2926	GCCACTGGTGTGGGCCATAATTCAATTCGC

These oligos produce a 388 base pair amplicon that was previously tested using an annealing gradient that indicates good amplification between 58°C and 68°C (ideally between 64° to 68 °C). This allows the use of the two-step PCR program described in Basic Protocol 1.

4% (w/v) Paraformaldehyde solution

Recipe for 1L: In 800-900 mL water, dissolve 4 g sodium hydroxide pellets, and then dissolve 40 g of PFA (this might take more than 20 min). Once the PFA is dissolved then add sodium phosphate (monobasic) until dissolved. Do not neutralize with sodium monophosphate too early because PFA will not dissolve in neutral solutions. Bring up to 1 L with water. Then filter the PFA using a vacuum or gravity filter to get rid of any residual PFA granules. Final pH should be about 7.4-7.5. Store at 4°C for 3-4 days. This recipe avoids unnecessary problems with the unfortunately common method of heating solutions to 60°C to dissolve PFA in neutral pH. PFA dissolves easily in alkaline solutions but not in neutral solutions.

30% (w/v) sucrose in PBS

Recipe for 1L: Dissolve 300 g of sucrose in 600 mL of 1X PBS. Then bring up to 1 L total volume with 1x PBS. Store at 4°C for 3 months.

20% Triton X-100

Recipe for 500 mL: Mix 100 mL of Triton X-100 with 400 mL of water. Triton X-100 is very viscous and will take time to mix with water. Store at room temperature for 6 months (or at 4°C if you observe any growth in the solution).

100 mM EGTA

Recipe for 500 mL: Add 19 g EGTA to about 200 mL of distilled water and add 10 M NaOH to dissolve. Then bring the volume up to 500 mL with water. Store at RT for 1 year.

Cryopreservant

Recipe for 1 L: Add 100 mL of 10x PBS into 400 mL water. Then add 200 mL glycerol and 20 mL DMSO. Then bring the volume up to 1 L with water. Store at 4°C for 1 year.

2x β-gal buffer

Recipe for 1 L:

-Add 27.6 g of $NaH_2PO_4 \bullet H_2O$ to 500 mL of distilled water (200 mM final). Use 10 M sodium hydroxide solution (about few mL) to bring pH to 7.4.

-Once dissolved add 11.7 g of sodium chloride (200 mM final).

-Then add 100 mL of 100 mM EGTA solution, followed by 4 mL of 1.0 M magnesium chloride solution (final concentration 4 mM), and then add 20 mL of 20% Triton X-100.

-Bring the volume up to 1 L with water. Final pH should be 7.4-7.5. Store at RT for 1 year.

Note: The pH of this stock solution buffer is critical to successful X-gal staining. If the pH is out of this range, the X-gal staining may not work. Make sure that the pH meter is calibrated properly.

X-gal solution

Recipe for 100mL, mix the following:

-50 mL of 2x β-gal buffer.

-2.5 mL of 200 mM $K_3Fe(CN)_6$ (0.165 g of $K_3Fe(CN)_6$ in 2.5 mL water)

-2.5 mL of 200 mM $K_4Fe(CN)_6 \bullet 3H_2O$ (0.211 g of $K_4Fe(CN)_6$ in 2.5 mL water)

-2.0 mL of 50 mg/mL X-gal dissolved in dimethyl formamide (DMF) (100 mg X-gal in 2 mL DMF)

-43 mL of water

This makes a solution with final concentrations of:

2.4 mM X-gal

100 mM sodium phosphate

100 mM sodium chloride

5 mM EGTA

2 mM $MgCl_2$

0.2% Triton X-100

5 mM $K_3Fe(CN)_6$

5 mM $K_4Fe(CN)_6$

Store away from light at 4 °C for 3-4 days or at -20 °C for 1 year.

Note: The final solution should have a yellowish color. Make sure to use DMF compatible tubes (opaque plastic).

COMMENTARY

Background Information

For many years neuroscientists have hypothesized that neuronal ensembles activated during a particular behavior played a direct role in mediating that behavior. However, techniques such as lesioning or pharmacological neuronal inactivation of whole brain areas or cell types lacked the ability to selectively silence only the behaviorally activated neurons. Daun02 inactivation allows us to determine whether Fos-expressing neuronal ensembles are necessary for many types of learned and motivated behaviors that are controlled by specific stimuli (typically conditioned stimuli that are associated with salient events such as drug-taking).

In many Daun02 studies performed so far, rats undergo behavioral training to associate two distinct sets of stimuli (e.g. drug reward and drug context 'A') via operant learning, such as lever pressing for a drug in a specific context (Bossert et al., 2011; Cruz et al., 2014a). After several conditioning sessions, the rats form a learned association (e.g. drug and drug context), and it is thought that this association is stored in neuronal ensembles in various brain areas (Cruz et al., 2013). On 'induction day', some day following the last training session, the rats are exposed to the conditioned stimulus (e.g. drug-associated context 'A') to activate a neuronal ensemble that encodes the learned association and modulates the associated behavior (e.g. drug-seeking) (see overview in Fig. 2). Daun02 is injected into the brain area of interest to silence this ensemble 90-120 min after ensemble activation, and then 3 days later on 'test day' the effects of Daun02 inactivation are examined by re-testing the animals for the same behavior that was observed on induction day. We have previously tested the idea that repeated presentations of the same stimuli activate the same neuronal ensemble (Bossert et al., 2011; Cruz et al., 2014a; Fanous et al., 2012; Koya et al., 2009). For example, if the rat is tested three days later by presenting the same stimulus as on induction day (e.g. drug-associated context A), then the associated behavior is disrupted. However, if Daun02 was injected following presentation of stimuli that was unrelated to the training context (e.g. a saline-associated context B or a novel context C), then drug seeking is not disrupted during testing in the drug-paired context A. This latter result indicates that Daun02 silenced a neuronal ensemble that is distinct from the neuronal ensemble that was activated by the drug-paired context A. This latter context or cue control is critical for demonstrating ensemble-specific inactivation and its effects on behavior. If this control for context or cue specificity is not included, then the effects of Daun02 inactivation on behavior cannot be distinguished from non-specific global inactivation methods such as

muscimol-baclofen or kainic acid. Of note, a novel context C often induces very high levels of Fos or β-gal expression in neurons unrelated to context A, which makes it a good control for demonstrating that it is the specific pattern of neurons that mediate behavior, and not the number of Fos or β-gal-expressing neurons. (Cruz et al., 2014a; Fanous et al., 2012)

Overall, Daun02 inactivation experiments require: 1) breeding sufficient numbers of *Fos-lacZ* rats, 2) β-gal expression in the brain area of interest following the behavior of interest, 3) successful guide cannula placement and Daun02 injections on 'induction day', 4) re-testing of the animals for altered behavior on 'test day'. A general overview of the Daun02 experimental timeline is shown in Figure 2.

Breeding *Fos-lacZ* rats

As previously mentioned, *Fos-lacZ* rats have been bred onto a Sprague-Dawley genetic background strain. However it is possible to backcross these *Fos-lacZ* rats onto different strains (e.g Long-Evans or Wistar). Using a conventional backcrossing procedure, it will take approximately 2.5 years to breed the Fos-lacZ transgene the required 10 generations onto a different strain. Companies such as Charles River may assist with 'speed congenics' services that require less time (1.5 years) to reach the same degree of congenicity. In practice, due to the extensive time it takes to generate a congenic animal, many researchers simply backcross for a few generations and utilize animals that are more than <90% genetically identical to the strain of interest. It is critical in these situations that pilot experiments are performed in order to assess whether these backcrossed animals phenotypically (e.g. on a behavioral level) resemble the strain of interest.

Describing the many different breeding protocols is beyond the scope of this protocol, and one may want to consult or leave this task to a breeding specialist at the animal unit in one's institute. But in short, a male or female that is heterozygous for the *Fos-lacZ* gene is crossed with an outbred wild-type male or female. Usually, 50% of the offspring (males and females) are heterozygous for the transgene, where as 50% are not. If studies are performed on only one sex, then only 25% of the offspring are useable for the Daun02 studies. As with all transgenic breeding procedures, excellent recordkeeping of the positive and negative offspring is crucial. Ideally, a specialist should manage the colony, genotyping, sorting positive and negative offspring, and record keeping, as these tasks become rather time-consuming when the colony sizes grow.

Critical Parameters

Designing the Daun02 experiment

Since Daun02 silences β-gal-expressing neurons, it is critical to check if the brain area(s) of interest expresses β-gal in *the Fos-lacZ* rats. We and others have observed that β-gal labeling is very weak or non-existent in many thalamic nuclei in *Fos-lacZ* rats, and these areas are not suitable for Daun02 inactivation (unpublished observations). To date, many

researchers have successfully used Daun02 in the prefrontal cortex and the striatum (Bossert et al., 2011; Cruz et al., 2014a; Koya et al., 2009; Pfarr et al., 2015). We have not tested this method in other brain areas that express high levels of β-gal such as the hippocampus or basolateral and central amygdala.

When designing the behavioral experiments it is important to include two main groups. In the first group, the aim is to induce β-gal using a 'known' stimulus (to induce the behavior of interest) to silence the neuronal ensemble of interest. For the second group, the aim is to induce β-gal in a different neuronal ensemble using a stimulus with different properties (e.g. visual, auditory) than the 'known' stimulus. Using these groups will allow one to determine whether the same stimulus repeatedly activates the same neuronal ensemble, and to determine whether distinct sets of stimuli activate different sets of ensembles.

Troubleshooting

X-gal staining

X-gal staining of brain tissue is easily influenced by the pH of the β-gal buffer, and hence it is important the pH probe is working optimally and/or the pH meter is properly calibrated. If the pH is incorrect (especially if it is acidic), then it is possible to see blue-stained blood vessels (possibly reflecting conversion of X-gal by mammalian β-gal in vessels) with little to no staining of β-gal-positive neurons. If blood vessel staining is observed, then remake the β-gal buffer. One can also use immunohistochemistry for Fos or β-gal protein expression.

Exclusion of animals from study

Although it is rare, PCR-based genotyping may produce false positives, i.e. a *Fos-lacZ*-negative animals may be labeled genotype-positive. In such cases, Daun02 will have no effect. Fortunately, these animals can be excluded by performing a X-gal stain or via β-gal immunohistochemistry. Also, as with any intracranial injection procedure, a small minority of injection sites may contain necrotic tissue. These animals should also be excluded.

Breeding pairs are not producing any transgene positive offspring

In such cases, re-genotype the transgene positive parent, and cull this animal if it turns out to be transgene negative. However, usually such cases can be avoided by taking two or more tissue samples and checking to see if there's consistency in the PCR results.

Anticipated Results

If Daun02 has silenced the neuronal ensemble of interest, then on 'test day' the associated behavior of interest will be altered compared to animals injected with vehicle or another group injected with Daun02 following exposure to a stimulus that should not activate the neuronal ensemble of interest (e.g. novel cue). If there is no Daun02-mediated behavioral alteration, there are several possibilities: 1) The neuronal ensemble in question does not directly mediate the behavior, but it could still play a general modulatory role (e.g. arousal) in the behavior. 2) Several neuronal ensembles can exist in the brain that encode the learned association, and inactivation of only one of these ensembles may leave the behavior largely intact.

Time Considerations

A considerable amount of time is required for breeding the *Fos-lacZ* rat colony. Each breeding cycle will take about 15 weeks for rats. This time frame includes time to reach sexual maturity (8-12 weeks), gestation period (3 weeks). Rats can be weaned usually at 3 weeks old, but for the purposes of implanting guide cannula, rats should be approximately 16 weeks old for their skulls to be thick enough to properly hold the cannulae. Also, the animals should recover for a week post-surgery. Thus, users should consider these time periods when planning experiments.

It would be advisable to consider about 4-6 months until a usable size colony is obtained. Also, a typical Daun02 experiment may require n=18-20 per group for 4 groups. As with all experiments requiring cannula, it is likely that 15-20% of animals will be excluded due to injection sites falling out of the target area. Our previous experience informs us that completion of a typical behavioral experiment with a 2x2 factorial design (e.g., test and control cues X Daun02 or vehicle) may easily take up to 1 to 1.5 years including breeding, surgeries, conducting behavioral experiments, performing X-gal staining, etc. Of course, this process may be sped up if the size of the breeding colony is expanded and/or multiple people simultaneously perform the surgeries and behavioral experiments.

ACKNOWLEDGEMENT (mandatory for NIH, optional for all others)

Eisuke Koya and Gabriella Margetts-Smith are funded by BBSRC grant number BB/M009017/1. Bruce Hope is funded by the NIDA Intramural Research Program, NIH.

LITERATURE CITED

Assi, H., Candolfi, M., Lowenstein, P. R., & Castro, M. G. . 2013. Rodent glioma models: intracranial stereotactic allografts and xenografts. Humana Press.

Bossert, J.M., Stern, A.L., Theberge, F.R., Cifani, C., Koya, E., Hope, B.T., and Shaham, Y. 2011. Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin. *Nat Neurosci* 14:420-422.

Cruz, F.C., Babin, K.R., Leao, R.M., Goldart, E.M., Bossert, J.M., Shaham, Y., and Hope, B.T. 2014a. Role of nucleus accumbens shell neuronal ensembles in context-induced reinstatement of cocaine-seeking. *J Neurosci* 34:7437-7446.

Cruz, F.C., Javier Rubio, F., and Hope, B.T. 2014b. Using c-fos to study neuronal ensembles in corticostriatal circuitry of addiction. *Brain Res*.

Cruz, F.C., Koya, E., Guez-Barber, D.H., Bossert, J.M., Lupica, C.R., Shaham, Y., and Hope, B.T. 2013. New technologies for examining neuronal ensembles in drug addiction and fear. *Nature Reviews Neuroscience* Nov;14(11):743-54.

Engeln, M., Bastide, M.F., Toulme, E., Dehay, B., Bourdenx, M., Doudnikoff, E., Li, Q., Gross, C.E., Boue-Grabot, E., Pisani, A., Bezard, E., and Fernagut, P.O. 2014. Selective Inactivation of Striatal FosB/DeltaFosB-Expressing Neurons Alleviates L-Dopa-Induced Dyskinesia. *Biol Psychiatry*.

Fanous, S., Goldart, E.M., Theberge, F.R., Bossert, J.M., Shaham, Y., and Hope, B.T. 2012. Role of orbitofrontal cortex neuronal ensembles in the expression of incubation of heroin craving. *J Neurosci* 32:11600-11609.

Farquhar, D., Pan, B.F., Sakurai, M., Ghosh, A., Mullen, C.A., and Nelson, J.A. 2002. Suicide gene therapy using E. coli beta-galactosidase. *Cancer Chemother Pharmacol* 50:65-70.

Fornari, R.V., Wichmann, R., Atsak, P., Atucha, E., Barsegyan, A., Beldjoud, H., Messanvi, F., Thuring, C.M., and Roozendaal, B. 2012. Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. *J Vis Exp* e3528.

Guzowski, J.F. 2002. Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. *Hippocampus* 12:86-104.

Herdegen, T. and Leah, J.D. 1998. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res Brain Res Rev* 28:370-490.

John, E.R. and Schwartz, E.L. 1978. The neurophysiology of information processing and cognition. *Annual review of psychology* 29:1-29.

Koya, E., Golden, S.A., Harvey, B.K., Guez-Barber, D.H., Berkow, A., Simmons, D.E., Bossert, J.M., Nair, S.G., Uejima, J.L., Marin, M.T., Mitchell, T.B., Farquhar, D., Ghosh, S.C., Mattson, B.J., and Hope, B.T. 2009. Targeted disruption of cocaine-activated nucleus accumbens neurons prevents context-specific sensitization. *Nat Neurosci* 12:1069-1073.

Ma, W., Rogers, K., Zbar, B., and Schmidt, L. 2002. Effects of different fixatives on beta-galactosidase activity. *J Histochem Cytochem* 50:1421-1424.

Morgan, J.I. and Curran, T. 1988. Calcium as a modulator of the immediate-early gene cascade in neurons. *Cell Calcium* 9:303-311.

Morgan, J.I. and Curran, T. 1991. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. *Annu Rev Neurosci* 14:421-451.

Paxinos, G.A.W.C. 1986. The rat brain in stereotactic coordinates. Academic, New York, U.S.A.

Pfarr, S., Meinhardt, M.W., Klee, M.L., Hansson, A.C., Vengeliene, V., Schonig, K., Bartsch, D., Hope, B.T., Spanagel, R., and Sommer, W.H. 2015. Losing Control: Excessive Alcohol Seeking after Selective Inactivation of Cue-Responsive Neurons in the Infralimbic Cortex. *J Neurosci* 35:10750-10761.

Rousselle, C., Clair, P., Lefauconnier, J.M., Kaczorek, M., Scherrmann, J.M., and Temsamani, J. 2000. New advances in the transport of doxorubicin through the blood-brain barrier by a peptide vector-mediated strategy. *Mol Pharmacol* 57:679-686.

Smeyne, R.J., Schilling, K., Robertson, L., Luk, D., Oberdick, J., Curran, T., and Morgan, J.I. 1992. fos-lacZ transgenic mice: mapping sites of gene induction in the central nervous system. *Neuron* 8:13-23.

Umemoto, M. and Olds, M.E. 1975. Effects of chlordiazepoxide, diazepam and chlorpromazine on conditioned emotional behaviour and conditioned neuronal activity in limbic, hypothalamic and geniculate regions. *Neuropharmacology* 14:413-425.

INTERNET RESOURCES

Helpful tips on tissue biopsy for genotyping

http://oacu.od.nih.gov/ARAC/documents/Rodent_Genotyping.pdf

FIGURE LEGENDS

Figure 1. Daun02-mediated selective inactivation of behaviorally activated neurons **A**) *Fos-lacZ* rat neurons express β-gal when activated. After Daun02 is locally injected in the target brain area, β-gal converts Daun02 into its active form, daunorubicin, which persistently reduces neuronal excitability and/or lesions neurons. **B**) Selective inactivation of behaviorally-activated neurons following Daun02 application.

Figure 2. A general overview of the experimental procedures beginning with breeding the *Fos-lacZ* animals and finishing with behavioral testing.

Figure 3. Diagram of skull surface indicating the position of the fissures, bregma, lambda, and potential bone screw positions. Figure modified from (Assi, 2013).

Figure 4. X-gal staining of *Fos-lacZ* rat brain. Note the dark blue β-gal expressing nuclei (red arrows).

TABLES (none)

Additional instructions:

The following should be submitted as individual files, NOT as part of the main document:

- Figures
- **COPYRIGHT PERMISSION** (all figures are new)

If you have any questions about your manuscript, or formatting, or submitting it, consult the Contributor's Style Guide (For Authors page) or contact the Developmental Editor listed for your title.

For questions about our submissions site (ScholarOne Manuscripts), your Contributor Agreement, or copyright permissions, please contact our Editorial Program Coordinator at cpsubmissions@wiley.com.